

Network-based integration of systems genetics data reveals pathways associated with lignocellulosic biomass accumulation and processing

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As a consequence of their remarkable adaptability, fast growth, and superior wood properties, eucalypt tree plantations have emerged as key renewable feedstocks (over 20 million ha globally) for the production of pulp, paper, bioenergy, and other lignocellulosic products. However, most biomass properties such as growth, wood density, and wood chemistry are complex traits that are hard to improve in long-lived perennials. Systems genetics, a process of harnessing multiple levels of component trait information (e.g., transcript, protein, and metabolite variation) in populations that vary in complex traits, has proven effective for dissecting the genetics and biology of such traits. We have applied a network-based data integration (NBDI) method for a systems-level analysis of genes, processes and pathways underlying biomass and bioenergy-related traits using a segregating *Eucalyptus* hybrid population. We show that the integrative approach can link biologically meaningful sets of genes to complex traits and at the same time reveal the molecular basis of trait variation. Gene sets identified for related woody biomass traits were found to share regulatory loci, cluster in network neighborhoods, and exhibit enrichment for molecular functions such as xylan metabolism and cell wall development. These findings offer a framework for identifying the molecular underpinnings of complex biomass and bioprocessing-related traits. A more thorough understanding of the molecular basis of plant biomass traits should provide additional opportunities for the establishment of a sustainable bio-based economy.

systems genetics | lignocellulosic biomass | cell wall | bioenergy | network-based data integration

Wood (secondary xylem tissue) from trees represents a significant proportion of global carbon sequestration (1, 2), while also providing raw materials, be it in the form of timber, paper, or other biomaterials and value-added derivatives, such as cellulose (3) and lignin (4). Fast-growing trees such as poplars and eucalypts with short rotation times, small genome sizes (500–600 Mbp), large genetic diversity, and established breeding populations are widely cultivated as woody biomass crops and are well suited for biotechnological improvement (5–8). A tree's amenability to bioprocessing for pulp, paper, cellulose, and other bio-products is dependent on the aggregate of its wood properties, which are a function of cellular architecture and the chemistry and ultrastructure of the secondary cell walls (SCWs) of wood fiber cells that compose the bulk of woody biomass (9). These properties are determined by overlapping developmental programs and pathways that have to be coordinated during secondary xylem development (xylogenesis) (10). Some of these pathways, SCW polysaccharide and lignin biosynthesis in particular, represent a strong, irreversible carbon sink and directly or indirectly use core metabolites (glucose/UDP-glucose and fructose) that are also used for growth and physiological/cellular homeostasis (energy metabolism, production

of amino acids, etc.). These metabolic interdependencies and the multitude of biological processes involved result in woody biomass traits having complex genetic architectures, especially in highly outbred organisms such as forest trees (11).

Functional genetics approaches based mainly on single-gene perturbations have been informative in revealing components of the SCW producing system in model plants (12). However, they have not necessarily yielded insight into the complex interactions of the genes that naturally affect cell wall chemistry and ultrastructure in ways that do not interfere with normal plant growth, form, and biomass accumulation. The characterization of natural genetic perturbations segregating in phenotypically wild-type individuals offers an attractive alternative tool to study properties that emerge from permissible genetic variation (11, 13). With the availability of high-throughput genotyping and high-resolution linkage maps in *Populus* (14, 15) and *Eucalyptus* (16–19), genetic approaches such as quantitative trait locus (QTL) (14, 20, 21) and LD-based association mapping (22–25) are becoming feasible to

Significance

Carbon fixation and accumulation as lignocellulosic biomass is of global ecological and industrial importance and most significantly occurs in the form of wood development in trees. Traits of importance in biomass accumulation are highly complex and, aside from environmental factors, are affected by many pathways and thousands of genes. We have applied a network-based data integration method for a systems genetics analysis of genes, processes, and pathways underlying biomass and bioenergy-related traits using segregating *Eucalyptus* hybrid tree populations. We could link biologically meaningful sets of genes to complex traits and at the same time reveal the molecular basis of trait variation. Such a holistic view of the biology of wood formation will contribute to genetic improvement and engineering of plant biomass.

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Table 1. Comparison of using NBDI-transformed versus using nontransformed expression values in prioritizing reference genes for 13 traits

Trait	NBDI enrichment p	Nontransformed enrichment p
DBH (over bark)	6.38×10^{-20}	2.81×10^{-11}
DBH (under bark)	5.55×10^{-15}	1.38×10^{-10}
Bark thickness	4.61×10^{-19}	1.38×10^{-10}
Wood density	6.52×10^{-10}	5.49×10^{-12}
Lignin content	2.83×10^{-01}	9.28×10^{-01}
Total C5 sugar in walls	1.02×10^{-01}	2.83×10^{-01}
Total C6 sugar in walls	1.02×10^{-01}	9.75×10^{-01}
Glucose released	5.49×10^{-12}	3.03×10^{-04}
Percent of max glucose released	3.29×10^{-14}	1.03×10^{-04}
Xylose released	1.88×10^{-13}	1.02×10^{-01}
% of max xylose release	2.21×10^{-03}	7.19×10^{-01}
Glucose + xylose released	2.81×10^{-11}	3.03×10^{-04}
Percent of max sugar released	3.29×10^{-14}	2.21×10^{-03}

For each trait, 300 genes were selected. The enrichment score corresponds to the P value of a hypergeometric enrichment test.

study woody biomass traits in long-lived perennial plants. While such association mapping approaches facilitate the delimitation of genome positions harboring causal variation (13), they provide little information as to how these genes and their variants act together in biological pathways to influence trait variation.

Complementing genetic information with molecular phenotypes (e.g., transcript levels) can contribute to a better mechanistic understanding of trait variation (24, 26, 27). Expression QTL (eQTL) analysis (28) allows the identification of genomic loci associated with variation in molecular phenotypes. In contrast to QTL or association analyses, eQTL analysis also identifies the genes that are affected by this variation and thus potentially contribute to the complex trait. However, because complex traits are subject to the combined effect of multiple genetic loci, each with a small effect on the trait, there is generally low power to detect statistically significant associations when relying on single-gene analysis. In addition, single gene associations do not show how genes and/or pathways interact to explain a complex trait (29).

To cope with the aforementioned limitations of the association problem, more integrated systems genetics approaches have been proposed (29, 30). Methods that use network models to represent molecular a priori knowledge on the organism/trait of interest (31) have been particularly successful to perform association analysis in clonal systems (32–35). In the context of outbred populations, network-based methods have been applied for gene prioritization (36) or to increase the reliability of eQTL association mapping itself (37) but not yet for integrative association analysis.

Here we applied a systems genetics approach to study the genomic loci and pathways affecting wood formation in *Eucalyptus*. We generated coupled genetics/genomics (linkage map and immature xylem transcriptome) data for 156 individuals segregating from an F2 pseudobackcross between a *Eucalyptus grandis* × *Eucalyptus urophylla* F1 interspecific hybrid tree and an unrelated *E. urophylla* tree and profiled traits representative of tree growth (diameter at breast height and bark thickness), wood properties (wood basic density and cell wall composition), and bioprocessing metrics (sugar release). Data were integrated using a unique network-based data integration (NBDI) approach that allows combining genotyping, expression profiling, and prior network information to prioritize genes and molecular mechanisms associated with complex wood formation traits.

Results

Network-Based Gene–Trait Association. One hundred fifty-six *E. grandis* × *E. urophylla* F2 interspecific backcross trees were profiled for transcript abundance in immature xylem and for woody biomass traits that relate to growth, wood density, cell wall composition, and sugar extractability (Table 1 and *Materials and Methods*). Genotyping, expression profiling, inferred eQTL associations, and prior network information were simultaneously used to prioritize genes and molecular mechanisms associated with each of the measured traits. To this end we developed an integration approach that makes use of a gene interaction network model in which nodes are genes and edges represent two types of information. If derived from prior information, edges reflect relations between genes and gene products, derived from Kyoto Encyclopedia of Genes and Genomes (KEGG) (*SI Materials and Methods*). If derived from eQTL associations, edges reflect that the connected genes share the same eQTL and thus are likely functionally related and coregulated.

This network model is then used to propagate on a per sample basis the expression signals of genes to a local network neighborhood. This propagation transforms the original gene expression data to network-diffused gene expression data (Fig. 1 and *Materials and Methods*). In the network-diffused expression matrix, each data point can be interpreted as the original expression signal of a gene in a sample, modulated by the expression of the genes that are close neighbors in the network (i.e., that are likely found in the same pathways or to share eQTLs, etc.). Modulation implies that if nodes in the local neighborhood of a gene are

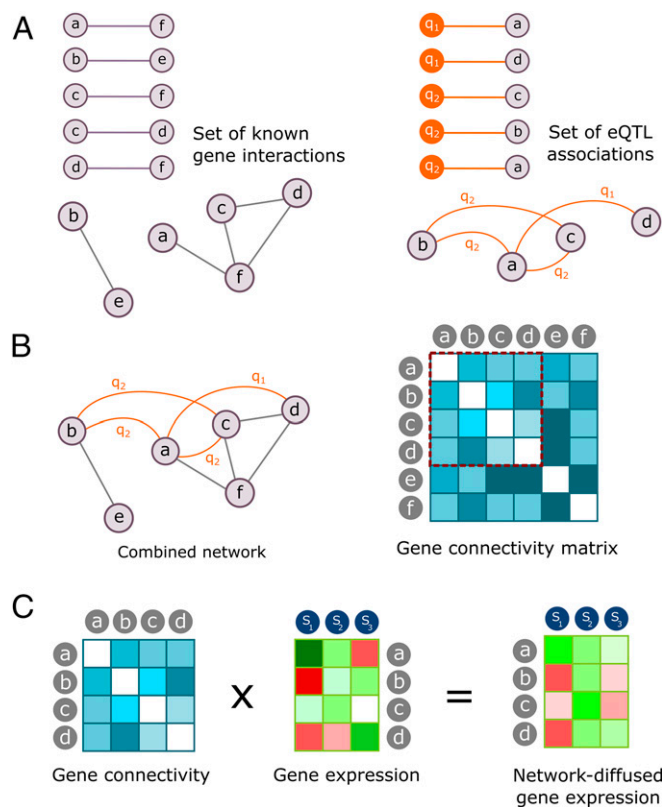


Fig. 1. An overview of the NBDI approach. Letters a–f represent genes, q_1 – q_3 are eQTL, and S_1 – S_3 are samples (xylem samples from individual trees). (A) Prior knowledge and eQTL associations are converted to a prior and functional interaction network, respectively. (B) The two networks are integrated to construct a combined network, and a gene–gene connectivity matrix is calculated (bright and dark colors show high and low connectivity, respectively). (C) The connectivity matrix (genes not present in the expression data are omitted) is used to diffuse gene expression data through the network.

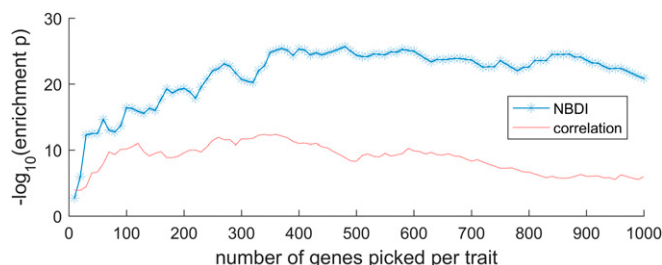


Fig. 2. Performance comparison of using NBDI-transformed (blue starred line) versus using nontransformed expression correlation data (red line) in prioritizing trait-associated genes. Enrichment probability (y axis; P value obtained using a hypergeometric enrichment test) of the combined gene selection (the union of all of the gene sets that were obtained per trait or, alternatively, the set of genes linked to at least one trait) is shown as a function of the number of genes prioritized per individual trait (x axis).

also expressed, the expression signal of the gene is confirmed and its relevance is increased; otherwise, its relevance is decreased. Using diffused gene expression is comparable to module-based analysis, where before further analysis, groups of genes with correlating gene expression (modules) are identified, under the assumption that these genes participate in the same biological processes or belong to the same pathway. The network neighborhood used to modulate the expression of a gene can be viewed as an implicit module of functionally related genes, with the main advantage of our approach that each individual gene can be prioritized or deprioritized for a particular trait based on the integrated information from network neighbors.

Once the gene expression values are diffused through the network to obtain the transformed expression values (referred to as NBDI-transformed values), genes relevant for each trait under study are identified by correlation analysis. Correlating NBDI-transformed values to each of the traits allows for ranking, per trait, of genes of potential relevance to that trait (for gene selections for each trait, see [Dataset S1](#)). To benchmark the performance of the NBDI approach, we compiled a literature-based set of reference genes with known biological roles in SCW biosynthesis (the best characterized and a central biological process in wood formation; [Dataset S2](#)) and calculated the extent to which gene sets associated with the traits were enriched for these reference genes. To illustrate the added value of using NBDI-transformed values, we also performed the same associations but using the nontransformed gene expression values when correlating gene expression variation

to trait variation. The difference in prioritization performance between our approach and that of using nontransformed expression values is illustrated as a function of the number of reference genes that is prioritized for either all traits together (Fig. 2) or for each individual trait (Fig. S1). Compared with using nontransformed expression data, NBDI-transformed data result in higher enrichment of reference genes, regardless of how many genes per trait were selected (Fig. 2). This higher enrichment can be explained by the fact that for most traits the NBDI transformation results in assigning a higher prioritization rank to reference genes than when using the nontransformed values (Fig. S2). In addition, with NBDI-transformed genes we were able to prioritize genes that associated to several traits as well as genes that were trait specific (Fig. S3). In the remainder of the analyses, we selected the 300 genes ([Dataset S1](#)) of which the NBDI-transformed expression correlated best with the trait. With this threshold a significant enrichment for reference genes (Table 1) was obtained, while still yielding results that were unique enough to explain trait differences. Applying these criteria resulted in a gene selection of 1,529 nonredundant genes ([Dataset S1](#)) that were linked to at least one of the traits and of which 102 were found in the reference set ($p_{\text{NBDI}} = 1.38 \times 10^{-21}$).

Genes and Pathways of Relevance to the Traits Under Study. The NBDI approach combines genetic and prior information with gene expression variation to prioritize, per trait, relevant genes/pathways influenced by genetic variation in the population. This combined information is captured in the two complementary views, an eQTL (Fig. 3) and a network view (Fig. 4).

First, because of its network model, NBDI implicitly imposes that genes prioritized for a trait should also share an eQTL at one or more loci in the genome. Most eQTLs for genes prioritized by the NBDI approach for a particular trait should therefore cluster together in hot spots rather than being randomly scattered along the genome. Fig. 3 shows this is indeed the case.

Second, as the NBDI approach favors genes that are connected in the interaction network, at least some of the genes associated with a trait, when projected on a gene interaction network, should cluster together and constitute a molecular subnetwork underlying the trait providing a complementary network view. Because this network of curated gene interactions (derived from KEGG) is sparser than the network used for the NBDI analysis that also includes eQTL overlap relations, by definition only a subset of the gene selections and relations can be visualized in subnetworks. To visualize eQTL relations for genes that could be connected through KEGG, eQTL overlap relations were overlaid on the identified subnetworks. For each trait, the largest connected component of the obtained network is

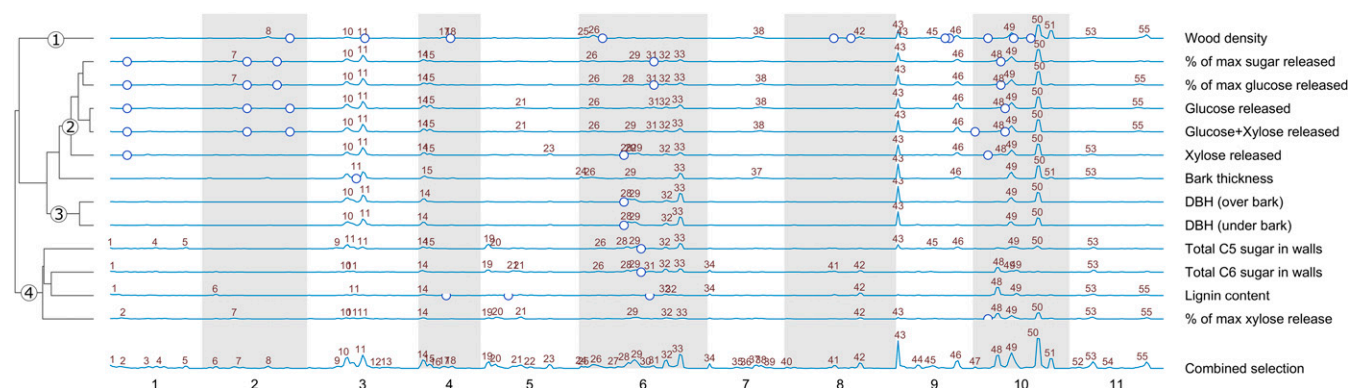


Fig. 3. Location of eQTLs shared by the genes selected for each individual trait. Height of the peaks represents the number of genes that have an eQTL in the same 5-cM-wide region (bin). Adjacent bins overlap 50%. Peak numbers correspond to the peaks identified for the combined gene selection (bottom row). Genes with eQTL in each peak are listed in [Dataset S3](#). Blue circles indicate previously identified trait QTLs. Traits are clustered based on the eQTL density profile ([SI Materials and Methods](#)). x axis labels are chromosome numbers. The white and gray vertical bars represent chromosomes. Circled numbers on the tree on the left correspond to the major groups of traits discussed in the main text.

Trait group 4 contains traits related to the total cell wall sugar content and, surprisingly, also lignin content and percent maximum xylose released. These traits are characterized by the relatively smaller effect of major eQTL peaks that dominate most of the other biomass traits. The lignin subnetwork (Fig. 4) in general lacks most of the genes related to lignin biosynthesis itself. Indeed, few association between the variation of expression of SCW biosynthetic genes (cellulose, xylan, and lignin pathway genes) and the final C5 and C6 sugar and lignin content of the cell wall were apparent (Dataset S3). Several genes highly associated with variation in lignin content code for enzymes involved in carbon metabolism, including phosphoenolpyruvate (phosphoenolpyruvate carboxykinase enolase), pyruvate (plastidial pyruvate kinase 3 and malate dehydrogenase), and acetyl-CoA (pyruvate dehydrogenase E1 α subunit) metabolism, as well as pathways producing UDP-glucose and fructose (UDP-glucose pyrophosphorylase and sucrose synthase; Dataset S3). Genes involved in mitochondrial energy metabolism were also associated with lignin content. Very distinctive for this group of traits are also the genes that relate to abiotic stress (eQTL peak 34) and RNA modification (a process associated with a very distinctive eQTL peak 48).

As an additional external validation, we overlaid previously identified QTLs (blue circles in Fig. 3) for the same complex traits (20) with the obtained eQTL frequency peaks (Fig. 3). These results show that at least some of these trait QTLs are in close proximity to eQTL frequency peaks (especially peaks 28, 29, 48, and 49, located on chromosomes 6 and 10), providing additional evidence that the gene selection is relevant for the trait under study. Several eQTL peaks cannot be directly mapped to trait QTLs. These might represent polymorphisms that only have detectable effects on molecular subcomponents of a trait but cannot be directly associated with the phenotype itself. Given that complex traits are affected by different molecular traits in epistatic and nonlinear ways, a direct link between molecular traits and phenotypic traits is not always expected or the effects are too numerous and small to detect at the level of trait QTLs given the relatively small size of experimental population ($n = 156$).

Discussion

The observed variability of woody biomass traits in this study is explained by the variation of combinations of genes or sets of closely interacting pathways influenced by genetic variation segregating in this particular interspecific backcross population. Because of this, linking a quantitative trait to the expression of individual genes might fail or be incomplete if the trait under study is influenced by variation in the expression of large numbers of genes that in turn can be influenced by the expression of other genes, etc. If this is the case, then any method that captures only the marginal effect of a gene on a trait might render only a partial view of the genes that are involved in the underlying biological processes. To cope with this statistical issue we have developed a network-based data integration approach (NBDI) that combines genotyping, expression profiling, and prior network information to prioritize genes and molecular mechanisms associated with measured traits.

This NBDI approach is based on a network model in which connections between genes reflect interactions derived from either prior molecular interaction information or from eQTL information. In the latter case it is assumed that if two genes share an eQTL, they are connected in the network because of a shared coregulation mechanism. Even though incidental overlap of eQTLs is possible, for instance, through the action of separate polymorphisms in tightly linked but unrelated genes, we assumed that the majority of the overlapping trans-eQTLs can be treated as evidence of a shared regulatory polymorphism, as reflected by the shared functional annotations observed for the associated genes. Gene expression signals are then propagated through the network model to obtain an integrated signal that is used to explain the variation in the external traits.

We applied the NBDI approach to study the genomic loci and pathways affecting wood formation in *Eucalyptus*. The experimental setup used [with high linkage disequilibrium (LD) and large effect QTLs segregating in a single family] is complementary to low-LD studies (with high resolution but typically small effect associations) in populations of unrelated individuals (e.g., refs. 24, 55).

Using our integrative systems genetics approach allowed for prioritizing genes contributing to woody biomass traits and identifying the putative regulatory loci with which these genes and traits are predominantly associated. Based on this analysis, a clear distinction could be made between growth and sugar release (bioprocessing) related traits and traits related to the total cell wall sugar content. Unexpectedly, we noticed little association between the variation of expression of SCW biosynthetic genes (cellulose, xylan, and lignin pathway genes) and the final C5 and C6 sugar and lignin content of the cell wall. Rather, most bona fide lignin biosynthesis genes were associated with growth-related traits (DBH and bark thickness), and most SCW polysaccharide biosynthesis genes were associated with variation in wood density and glucose release efficiency. Several genes highly associated with variation in lignin content code for enzymes involved in carbon metabolism and in mitochondrial energy metabolism. As a result, we hypothesize that variation in the expression of SCW biosynthetic genes has an effect on the growth and ultrastructure and resultant processability of the secondary cell wall, whereas the quantity of sequestered carbon in the cell wall (in the form of polysaccharides and phenolics) is more related to variation in primary carbon metabolism pathways and hence precursor availability. This assumption further establishes the strong link between physiological/cellular homeostasis and secondary processes such as SCW polysaccharide and lignin biosynthesis that represent a strong, irreversible carbon sink in woody plants.

Materials and Methods

Experimental Population, Transcriptome, and Complex Trait Analysis. The F2 backcross population was generated from a cross between an *E. grandis* \times *E. urophylla* F1 interspecific hybrid parent (GUSAP1, Sappi Forest Research, South Africa) and an unrelated *E. urophylla* parent (USAP1) (18). At 3 y old, immature xylem tissue was harvested from 156 individuals as previously described (56). Samples were collected from 3-y-old trees over a 7.5-h period between 0900 and 1630 hours for 3 d. Total RNA was isolated (57) and used for RNA-Seq expression profiling (30 million; Illumina PE50, BGI Hong Kong). Gene expression values (FPKM) were calculated per gene model using TopHat version 1.3 and Cufflinks version 1.0.3 (bias correction and quartile normalization was enabled for the FPKM calculation) (58, 59). Diameter (cm) at breast height (DBH) of the main stem was assessed as described previously (20). Bark thickness was calculated as the difference between over-bark and under-bark DBH measurements. A wood disk taken at breast height (1.35 m) was used to determine wood basic density using the water displacement method (www.tappi.org/content/SARG/T258.pdf). Chemical wood properties were assessed using different analytical methods, including pyrolysis molecular beam mass spectrometry (pyMBMS).

Trait QTL mapping, eQTL mapping, and eQTL classification are described in *SI Materials and Methods*.

NBDI Association Analysis. First, a hybrid gene interaction network was constructed using curated gene interactions downloaded from KEGG and eQTL overlap relations (Fig. 1A). For the latter, we investigated for pairs of genes whether these genes had overlapping eQTL intervals (Fig. S5). If this is the case, a connection in the hybrid network is added. Once the network is constructed, a graph node kernel was calculated (the Laplacian exponential diffusion kernel; 60) to quantify how well each node in the network connects to all other nodes (Fig. 1B). The resulting connectivity matrix was then multiplied with the gene expression matrix to obtain the diffused or transformed gene expression matrix (Fig. 1C). Genes in the network connectivity matrix that were not present in the gene expression matrix were removed and vice versa. The transformed gene expression was finally linked to the measured traits by calculating the absolute value of the Pearson correlation between the transformed gene expression and the measured traits. After ranking, the top 300 genes exhibiting the highest correlation were selected for further analysis. For details of the eQTL overlap procedure, network construction, connectivity calculation, and association analysis, see *SI Materials and Methods* and Figs. S5 and S6.

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